Distinct cellular effects and interactions of the Rho-family GTPase TC10

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Background: Rho-family GTPases have central roles in cytoskeletal organization, proliferation, differentiation and apoptosis. Multiple factors possessing overlapping specificities for Rho GTPases have been identified. The Rho GTPases Cdc42 and Rac share many regulators and effectors, yet produce different phenotypes when expressed as gain-of-function mutants in cells. The Rho-family member TC10 has remained almost completely uncharacterized, so it was of interest to determine whether TC10 has unique cellular effects and interacts with the same targets as Cdc42 and Rac.

Results: A gain-of-function TC10 mutant protein expressed in fibroblasts induced cell rounding, loss of stress fibers and formation of peripheral extensions. The extensions were longer than those induced by the analogous Cdc42 mutant protein. Cells expressing TC10 also possessed fewer membrane ruffles and stress fibers than those expressing Cdc42. *TC10* mRNA was most highly expressed in heart and skeletal muscle. The GTPase activity of TC10 was lower than that of Cdc42, and TC10 possessed a lower affinity for, but greater responsiveness to, the p50Rho GTPase-activating protein (p50RhoGAP) than did Cdc42. TC10 stimulated Jun N-terminal kinase (JNK) and p21-activated kinase (PAK) activities and interacted with a set of effectors (α-, β- and γPAK, MRCKα/β, MLK2, N-WASP and MSE55) that overlaps with those for Cdc42 and Rac. TC10 did not interact with MLK3 or WASP, and interacted only weakly with ACK-1.

Conclusions: TC10 possesses distinct features, but exhibits a phenotype most closely related to that of Cdc42. It interacts with a similar subset of effectors to Cdc42 but not with MLK3, WASP or ACK-1. It is regulated differentially by p50RhoGAP.

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Background

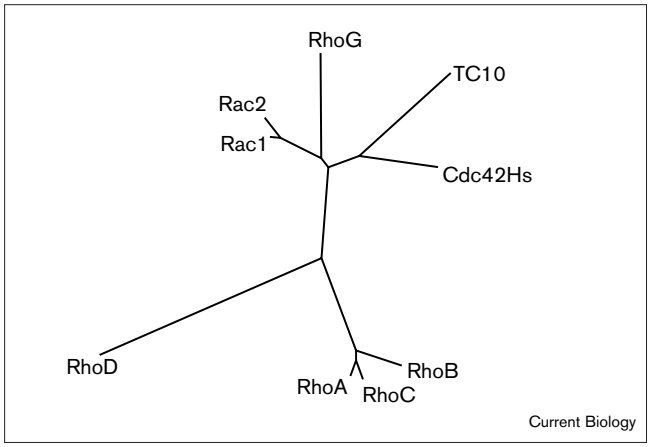
The Rho family of small GTPases includes RhoA, B, C, D, E and G, Rnd1 and Rnd2, Rac1 and Rac2, RacE, Cdc42 and TC10 [1–4]. These proteins act as molecular switches that control many cellular processes, including cytoskeleton remodeling, gene expression, cell proliferation, invasion, differentiation and apoptosis [4–6]. Their activities are positively regulated by guanine-nucleotide exchange factors (GEFs), which promote exchange of GDP for GTP, and are negatively regulated by GTPase-activating proteins (GAPs), which increase intrinsic GTPase activity. TC10 was originally cloned from a human teratocarcinoma cDNA library [7]. The TC10 protein is most similar to Cdc42 and Rac1 (Figure 1), but is unique in possessing short extensions at the amino and carboxyl termini.

Introduction of gain-of-function (GTP-bound) mutants of Rho into fibroblasts results in the formation of stress fibers and focal adhesions [8]. Equivalent mutations in Rac1 produce membrane ruffles and focal complexes [9,10] and in Cdc42 induce filopodia, microspikes and focal complexes [10,11] and reduce stress fiber density [11].

Rho, Rac, and Cdc42 have also been implicated in cellular processes distinct from cytoskeletal rearrangements. They stimulate cell-cycle progression and DNA synthesis and are involved in Ras-mediated transformation [12–17]. Rac and Cdc42 also stimulate phosphatidylinositol 3-kinase, Jun N-terminal kinase (JNK)/stress-activated protein kinase, and p38/Mpk1 mitogen-activated protein kinase pathways [5,13,18–20]. The activation of JNK is independent of effects on the actin cytoskeleton [21].

Given the similarities between Rho-family GTPases, the structural basis for their different physiological effects remains to be established. Therefore, an important goal is to identify the complete set of proteins that interact with these GTPases and to determine their specificities. Rho-family GTPases are regulated by GAPs, including p50GAP (p50RhoGAP or Cdc42GAP), and the Dbl family of GEFs [4]. There are also many effectors for Rho-family GTPases.

Figure 1



Phylogenetic tree of human Rho-family members. An unrooted distance tree showing the relationships among the translated human Rho-family sequences, constructed with the PHYLIP 3.5c phylogenetic inference package of Felsenstein (University of Washington) using the Fitch and Margoliash method. Branch lengths are drawn to scale, with longer branches indicating more changes. TC10 has 67.4% and 64.2% amino-acid identity to human Cdc42 (Cdc42Hs) and Rac1, respectively.

Effectors of one large family contain a CRIB (Cdc42/Rac interactive binding) domain, which is a 16-residue sequence with eight core amino acids [22]. Proteins possessing this motif include the kinases α-, β-, and γPAK, MRCKα/β, MLK2/3 and ACK-1/-2, Wiskott–Aldrich syndrome protein (WASP), N-WASP and MSE55 [22–25]. There are also several non-CRIB-containing targets of Cdc42 and Rac, such as IQGAP1/2 [3]. Most of these targets bind only to GTP-associated Cdc42 and Rac and many have overlapping specificities, although several interact with only one of the two GTPases. Here, we have investigated the properties of TC10, including its regulation, interactions with effectors and effects on the actin cytoskeleton.

Results

To determine the cytoskeletal effects of TC10, a gain-of-function mutant, TC10(Q75L), was created and transfected into NIH3T3 fibroblasts. An amino-terminal, triple-hemagglutinin (HA) tag was attached to facilitate detection. For comparison, parallel cultures were transfected with gain-of-function mutants RhoA(G14V), Rac1(G12V) and Cdc42(Q61L); see Figure 2. RhoA(G14V) increased actin stress fibers, Rac1(G12V) produced peripheral membrane ruffling and Cdc42(Q61L) induced peripheral extensions. TC10(Q75L) also produced peripheral extensions, but the phenotype was more dramatic, with longer extensions in a greater percentage of cells (Table 1). Time-lapse images of cells expressing TC10(Q75L) demonstrated that the phenotype is due to extensions rather than retractions (data not shown). Stress fibers were undetectable in most cells expressing TC10(Q75L), whereas Cdc42(Q61L) had no effect or only slightly decreased stress fibers compared with control cells expressing green fluorescent protein (GFP). Cdc42(Q61L) also consistently produced membrane ruffling; cells that expressed TC10(Q75L) had only small membrane ruffles, similar to those in cells expressing GFP. The differences in phenotype between TC10(Q75L) and Cdc42(Q61L) were statistically significant (Table 1). TC10(Q75L) induced peripheral extensions that were on average 67% longer than those induced by Cdc42(Q61L). Cells expressing wild-type TC10 had a phenotype similar to that of controls (data not shown).

A trivial explanation for these morphological differences might be that TC10(Q75L) protein was expressed at higher levels than Cdc42(Q61L). We therefore transfected cells with a range of plasmid concentrations and determined expression levels. High concentrations of Cdc42(Q61L) reduced the transfection efficiency and total expression level (data not shown), suggesting that ectopic expression of Cdc42(Q61L) can cause cell detachment and/or cell death. In contrast, TC10(Q75L) did not appear to be toxic, even at high concentrations. To overcome this problem, we quantitated expression on a single-cell basis by measuring the total fluorescence intensity of each cell immunostained with anti-HA antibody. These data show that similar levels of ectopically expressed HA–Cdc42(Q61L) and HA–TC10(Q75L) produce distinct phenotypes (Table 1).

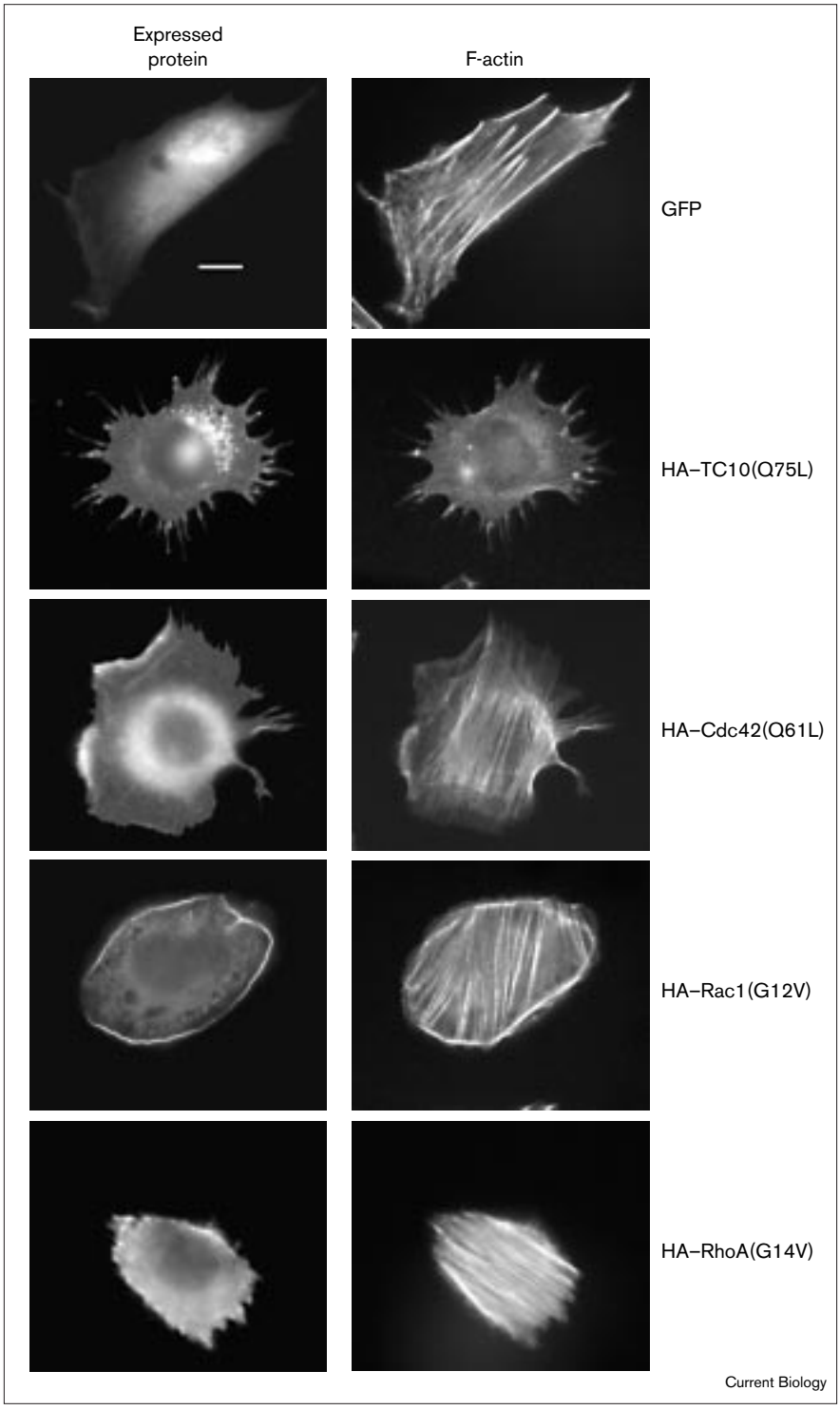
To compare the intracellular locations of wild-type TC10 and Cdc42, we co-expressed HA-tagged TC10 with Myc-tagged Cdc42 (Figure 3) or Myc-tagged TC10 with HA-tagged Cdc42 (data not shown) proteins in NIH3T3 cells. The HA-tag produced a more granular appearance than did the Myc-tag, but both proteins were located around the nucleus. TC10 was usually concentrated on one side of the nucleus and appeared punctate or vesicular; Cdc42 appeared more diffuse.

To determine whether the *TC10* and *Cdc42* genes are co-expressed in tissues, we examined the expression patterns of *TC10* and *Cdc42* in several tissues by northern blot analysis. *TC10* expression was markedly elevated in heart and skeletal muscle and was low in all other tissues (Figure 4), whereas *Cdc42* was expressed at intermediate levels in all tissues examined.

One important difference between TC10 and Cdc42 proteins may be the regulation of their GTPase and GTP-binding activities. To investigate this, we produced TC10 protein in *Escherichia coli* (Figure 5a) and compared its basal nucleotide exchange and GTPase activities with those of Cdc42. The GTP off-rates for TC10 and Cdc42 at 30°C were similar, with K_{off} values of 0.018 ± 0.002 (mean ± standard deviation; SD) and 0.009 ± 0.001, respectively (Figure 5b). However, the intrinsic GTPase activity of TC10 was fivefold lower than that of Cdc42 (Figure 5c).

Figure 2

Cell phenotypes of Rho-family members. NIH3T3 cells were transfected with 2 μg pKH3-TC10(Q75L), pKH3-Cdc42(Q61L), pKH3-Rac1(G12V), or pKH3-RhoA(G14V) on 2 cm^2 Lab-Tek slides. Cells expressing triple-HA-tagged proteins were visualized with 12CA5 anti-HA monoclonal and Cy3-conjugated anti-mouse antibodies. F-actin was stained with FITC–phalloidin. pRK7-GFP was transfected as a negative control, and in this case F-actin was stained with TRITC–phalloidin. Images were captured with a Nikon inverted microscope with a 60× water immersion lens and a Hamamatsu charge-coupled device (CCD) camera. Images were processed with Improvision Openlab and Adobe Photoshop software. The scale bar represents 10 μm.



To investigate the regulation of these two GTPases further, we compared their responses to p50GAP, a Rho-family GAP with a preference for Cdc42. The GAP domain of p50GAP was expressed as a glutathione-S-transferase (GST) fusion protein in *E. coli*. The data are presented as k_{cat} values for TC10 and Cdc42 (Figure 6a) or as fold activation (ratio of the GAP-catalyzed rate constant to the intrinsic catalytic rate constant) of TC10 and Cdc42 GTPase

Table 1

Quantitation of TC10(Q75L) and Cdc42(Q61L) phenotypes in NIH3T3 cells.

	GFP control	TC10(Q75L)	Cdc42(Q61L)
Spiked cells (%)			
without stress fibers	2.5 ± 1.5	78 ± 3†	25 ± 6†
with stress fibers	3.4 ± 1.3	8 ± 0.4†	38 ± 3†
No. of spikes per cell*	ND	38 ± 2‡	21 ± 2‡
Spike length*	ND	4.5 μm ± 0.2‡	2.7 μm ± 0.2‡
Cell fluorescence*	ND	2035 ± 180	2715 ± 344

NIH3T3 cells were transfected with 2 μg pKH3 DNA on 2 cm^2 Lab-Tek slides. Cells expressing triple-HA-tagged proteins were visualized with 12CA5 anti-HA and Cy3-conjugated anti-mouse antibodies. Simultaneously, F-actin was visualized with FITC–phalloidin. Control cells were transfected with pRK7-GFP and stained with TRITC–phalloidin. Cell phenotypes were quantitated by counting more than 100 cells in each of two experiments. Spike length, number, and total cell fluorescence were measured and quantitated with Improvision Openlab software. The *p* values were determined by unpaired Student *t* tests. Values are mean ± SEM of two experiments or $*n = 20$. ND indicates not determined. †Significant difference, *p* value < 0.02. ‡Extremely significant difference, *p* value < 0.001.

activity (Figure 6b) as functions of GST–GAP concentration. The GTPase activities of TC10 and Cdc42 were activated to similar levels by GST–p50GAP, but the fold stimulation of TC10 was substantially higher; Cdc42 had an approximately 10-fold higher apparent affinity for p50GAP than had TC10. As expected, TC10(Q75L) protein was insensitive to inactivation by GST–p50GAP (data not shown). These results suggest that regulated inactivation of TC10 is significantly different from that of Cdc42.

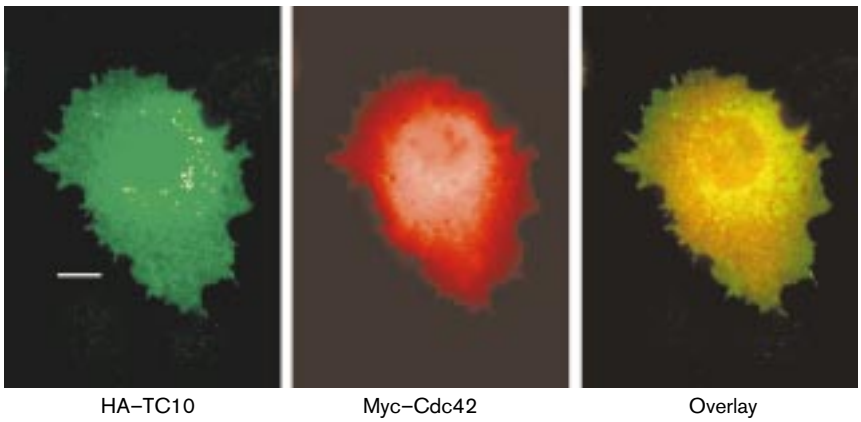
We next examined the interactions of TC10 with Rho-family effectors. In an overlay assay, TC10(G26V) and Cdc42 bound to Myc-tagged αPAK and to a Glu–Glu-tagged RasGAP-related domain of IQGAP1 (Figure 7a); binding was GTP dependent. Relative affinities of TC10 and Cdc42 for αPAK were similar (TC10 = 3.3 nM, Cdc42 = 2.9 nM, data not shown).

Next we examined whether TC10 can activate PAK and JNK activities, as can Cdc42 [13,18–20,26–28]. GTP-bound, recombinant TC10 and Cdc42 both stimulated αPAK (Figure 7b), and Myc-tagged TC10(Q75L) or Cdc42(Q61L) stimulated HA–JNK activity when co-expressed *in vivo* (Figure 7c).

The distinct morphologies induced by gain-of-function TC10 and Cdc42 mutants suggest that the sets of effectors cannot be identical. Therefore, we performed a saturating yeast two-hybrid screen of a total mouse embryo (day 9) library to identify TC10-interacting proteins [29]. As bait, we used TC10(Q75L) lacking the carboxy-terminal prenylation motif and fused at its amino terminus to the GAL4 DNA-binding domain. Among the 6.5×10^6 transformants screened, 368 clones grew on selective medium. Of these, approximately two-thirds selectively activated the *lacZ* reporter gene in the presence of TC10(Q75L) bait plasmid. Plasmid DNAs were prepared from 60% of positives and analyzed by sequencing or PCR. Ninety-five clones encoded eight known CRIB-domain-containing effectors of Cdc42: α-, β- and γPAK, MRCKα/β, MLK2, N-WASP and MSE55. Our partial sequences for the 25 MRCKβ clones were all identical but differed from the published rat amino-acid sequence (corresponding to bases 4884–4947 and after position 5113); these differences appear to result from frameshift errors in the published sequence [25].

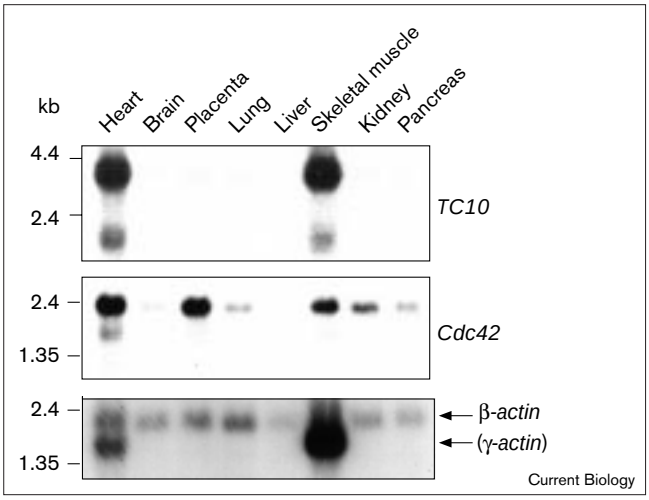
To confirm the ability of TC10 to interact with these effectors, plasmids in yeast strain HF7c (MAT**a**) were tested by conjugation against W303 (MATα) transformed with empty vector or plasmids encoding GAL4 fusions of

Figure 3



Co-localization of TC10 and Cdc42 proteins. NIH3T3 cells were transfected with 1 μg pKH3-TC10 and pKMyc-Cdc42 on 2 cm^2 Lab-Tek slides. Triple-HA-tagged proteins were detected with polyclonal anti-HA and FITC-conjugated anti-rabbit antibodies. Myc-tagged proteins were detected with monoclonal 9E10 anti-Myc and Texas-red-conjugated anti-mouse antibodies. Images were captured and processed as in Figure 2. The scale bar represents 10 μm.

Figure 4



Comparison of *TC10* and *Cdc42* RNA expression in human tissues. A poly(A)$^+$ RNA multiple tissue northern blot (Clontech) was probed with [^{32}P]dCTP-labeled coding regions of *TC10*, *Cdc42* or *β-actin*. The lower band in the *β-actin* control represents cross-hybridization with *γ-actin*. Size markers (in kb) are shown to the left.

TC10(Q75L), Cdc42(Q61L), Rac1(G12V), RhoA(G14V), Rab3A(Q81L) or the inactive mutant TC10(T31N). All diploids expressing TC10(Q75L) or Cdc42(Q61L) grew on selective medium (Figure 8a). As previously described [22,27], all three PAKs and MLK2 also showed strong interactions with Rac1, but not with RhoA or Rab3A. MRCKα/β, N-WASP and MSE55 did not interact strongly with Rac1 in this assay.

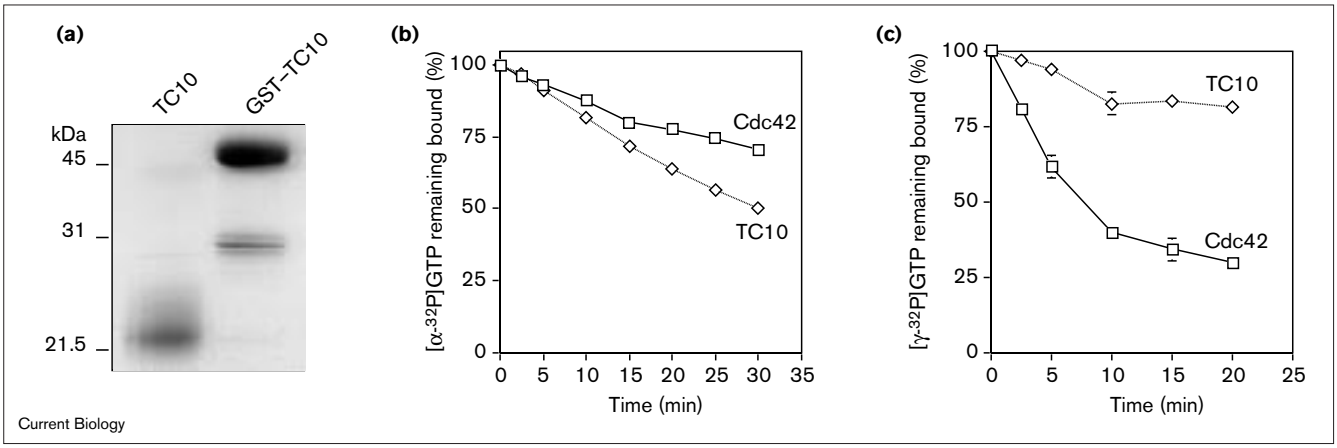
The only known CRIB-domain-containing proteins that were not isolated in our screen were MLK3, WASP and ACK-1/-2. We reasoned that either these genes are not represented in our library, or the encoded proteins do not interact efficiently with TC10. To distinguish between these possibilities, we obtained partial clones of MLK3 and WASP and tested them in the yeast two-hybrid system. Remarkably, although MLK3 and WASP were positive for interaction with Cdc42(Q61L), they were negative for TC10(Q75L) (Figure 8b). As positive controls, MLK2 and N-WASP were shown to interact with TC10 and Cdc42. We also expressed a GST fusion of the Cdc42-interacting domain of ACK-1. In an overlay assay, GST–ACK-1 interacted very weakly with TC10 but strongly with Cdc42 (Figure 8c).

Discussion

TC10 is a previously uncharacterized member of the Rho-family of small GTPases. Other family members have key roles in cytoskeletal organization, cell-cycle progression and activation of signal transduction cascades. An abundance of regulators or effectors has been identified for these GTPases. Here we have provided the first characterization of TC10 and have shown that it possesses several unique properties that distinguish it from other Rho-family members.

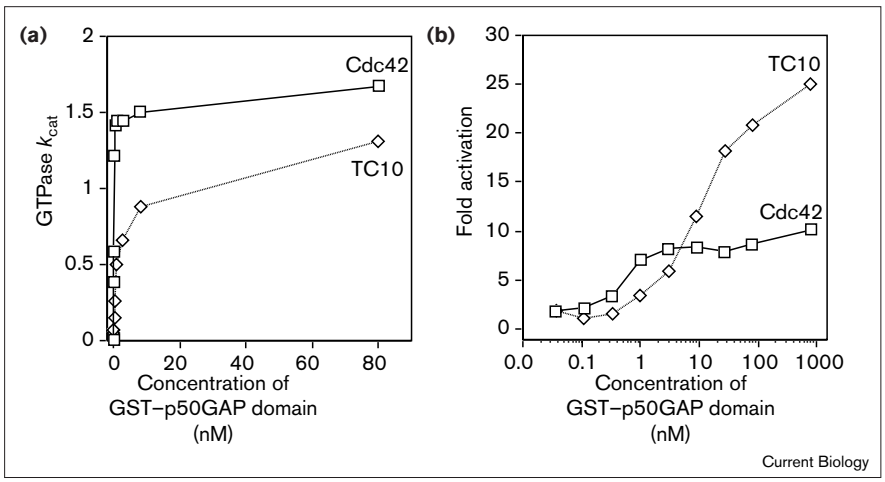
TC10, Cdc42 and Rac possess nearly identical sequences within the Switch I and Switch II domains that participate

Figure 5



Characterization of TC10 protein. (a) Coomassie-stained SDS–PAGE of recombinant GST–TC10 and TC10. GST–TC10 was expressed in *E. coli*, purified on glutathione–Sepharose and eluted with glutathione or cleaved with thrombin. Molecular weight markers (in kDa) are shown to the left. (b) Release of GTP from TC10 and Cdc42. Protein (3 μg) was loaded with 5–10 μCi [α-^{32}P]GTP and incubated at 30°C. Aliquots were removed at intervals and bound nucleotide was quantitated after nitrocellulose filter binding. (c) Intrinsic GTPase activity of TC10 and Cdc42. Protein (3 μg) was loaded with 5–10 μCi [γ-^{32}P]GTP; bound nucleotide remaining with respect to time was measured as in (b). GTP hydrolysis was calculated after subtraction of GTP release using the data from (b). In (b,c), values are the mean of two experiments; error bars represent standard deviation.

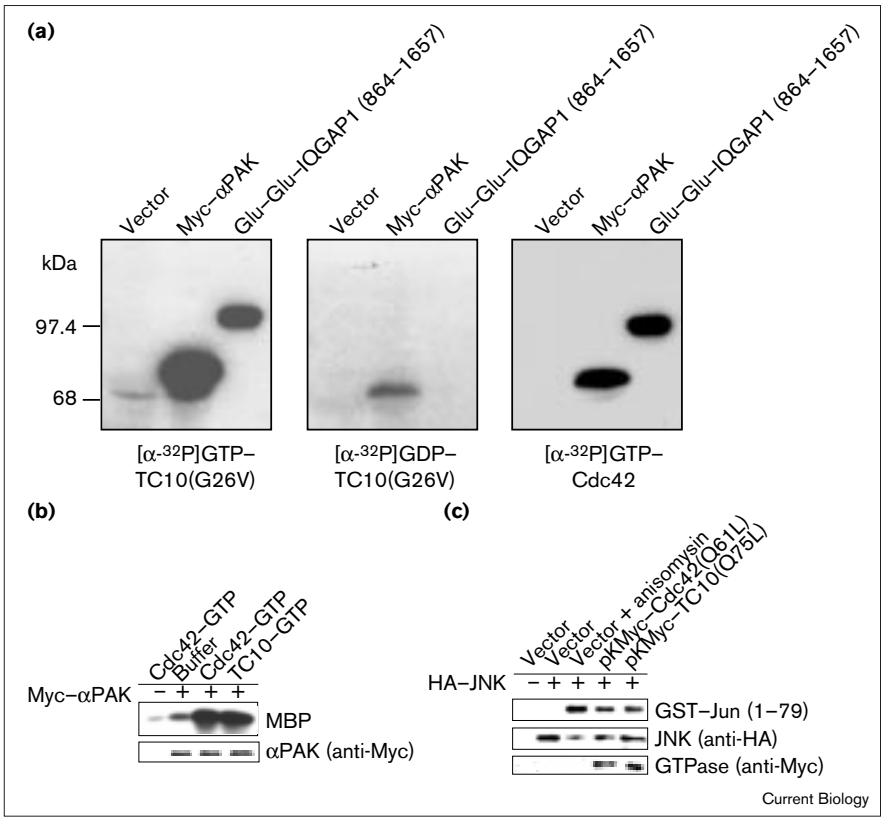
Figure 6



Comparison of p50GAP activity on TC10 and Cdc42. Various concentrations of GST–p50GAP (GAP domain; amino acids 864–1657) were incubated at 30°C for 3 min with [γ-^{32}P]GTP-loaded TC10 or Cdc42 (0.6 μg). Reactions were terminated by nitrocellulose filter binding. (a) Intrinsic k_{cat} values were calculated assuming single-exponential kinetics. (b) Fold activation was calculated as the ratio of the k_{cat} in the presence of GAP to the intrinsic k_{cat}. Values shown are the mean of two experiments; similar results were obtained in four independent experiments.

in binding to regulatory factors and effectors. Cdc42 and Rac activate JNK, bind to proteins containing a CRIB domain, and can be controlled by a common subset of regulators. Nevertheless, each can interact with distinct exchange factors and effectors, and each stimulates distinct changes in cell morphology. Therefore, we expected

Figure 7



Interaction of selected Rho-family effector proteins with TC10. (a) Effect of the nucleotide-bound state of TC10 on interactions with αPAK and IQGAP1 by overlay assay. Myc–αPAK was expressed in COS-7 cells (10 μg DNA per 100 mm plate). Extracts from pKH3-transfected cells (vector) were used as a negative control. For Glu–Glu-tagged IQGAP1 (864–1657) 3 μg was loaded per lane. Proteins were transferred to nitrocellulose and probed with equal specific activities of [α-^{32}P]GTP-TC10(G26V), [α-^{32}P]GDP-TC10(G26V) or [α-^{32}P]GTP-Cdc42. (b) Activation of αPAK by TC10. COS-7 cells were transfected with pCMV6M-αPAK (5 μg DNA per 100 mm plate) then serum-starved overnight. Myc–αPAK was immunoprecipitated with anti-PAK antibody, stimulated with 5 μg GTP-bound TC10 or Cdc42, and incubated at 30°C with 5 μg myelin basic protein (MBP) and 5 μCi [γ-^{32}P]ATP. Reactions were analyzed by autoradiography after SDS–PAGE. Immunoprecipitated proteins were quantitated by immunoblot analysis with 9E10 antibody (anti-Myc; lower panel). (c) Activation of JNK activity by TC10. COS-7 cells were co-transfected with 1 μg pKH3-JNK and 5 μg pKMyc-TC10(Q75L) or pKMyc-Cdc42(Q61L) per 100 mm plate and serum-starved overnight. Anisomysin at 10 μg/ml was used as a positive control for JNK activation. HA–JNK was immunoprecipitated with 12CA5 antibody and incubated at 30°C with 2 μg GST–Jun (1–79) and 2 μCi [γ-^{32}P]ATP. Reactions were analyzed by autoradiography after SDS–PAGE. Expressed proteins were quantitated by immunoblot analysis with 12CA5 (anti-HA) and 9E10 antibodies (lower panel). In (b,c), data represent one of three independent experiments.

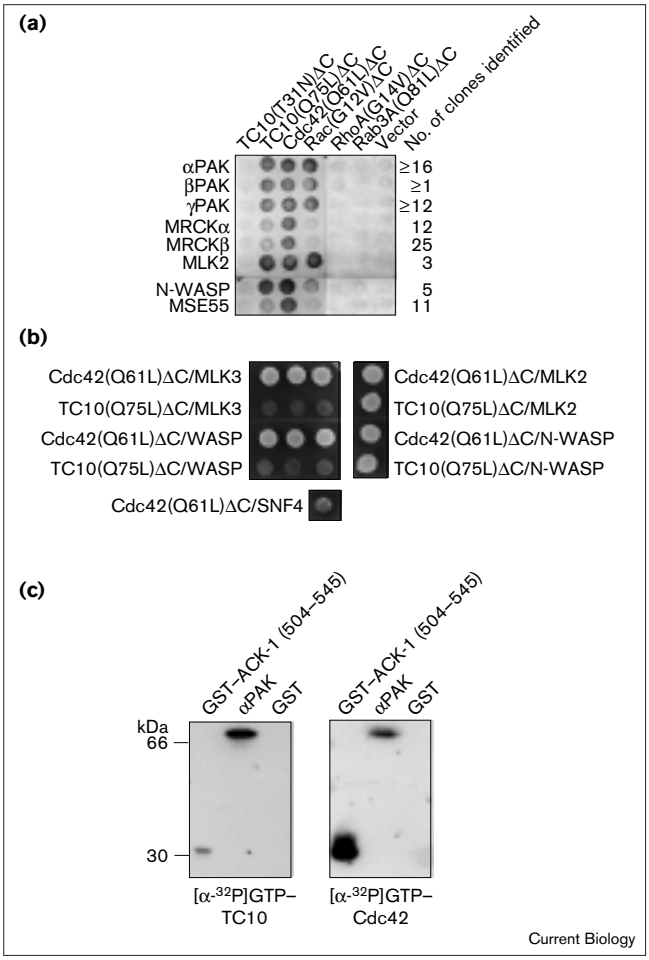
that TC10 might possess unique properties and cellular functions despite sequence similarities to other family members. Indeed, when a dominant gain-of-function TC10 mutant was expressed in NIH3T3 cells, a striking phenotype was induced, with long peripheral extensions and the complete loss of stress fibers in most of the cells. There were also few membrane ruffles. The exaggerated filopodial phenotype is reminiscent of that induced by ectopic co-expression of N-WASP with gain-of-function Cdc42(G12V) [24]. N-WASP interacted with TC10 in the two-hybrid assay.

No TC10 homolog is present in budding yeast, and we predicted, therefore, that it might possess a function specific to differentiated cells. As a first test of this hypothesis, we examined *TC10* mRNA levels in several tissues and found markedly high levels in heart and skeletal muscle. While *Cdc42* was also expressed in heart and skeletal muscle, the difference between tissues was not so striking as for *TC10*. Recently, it was reported that Rho-family members may have a role in muscle differentiation; gain-of-function Rho, Rac and Cdc42 mutants can stimulate transcription of muscle-specific genes required for differentiation [30]. Within the cell, TC10 and Cdc42 both appear around the nucleus, but TC10 appears more vesicular with localization to one side of the nucleus; Cdc42 appeared more diffuse than in the report of Erickson *et al.* [31] where Cdc42 was found to be localized to the Golgi apparatus.

The intrinsic guanine nucleotide off-rate of TC10 is twice that of Cdc42, and the basal GTPase rate is approximately fivefold lower. In the absence of regulatory factors, these differences would lead to a higher proportion of TC10 being in the GTP-bound active conformation within the cell. To test whether these differences might be mitigated or exaggerated by regulatory factors, we determined the effects of p50GAP. Interestingly, TC10 possesses a lower apparent affinity for p50GAP than does Cdc42, so that at low concentrations of p50GAP, the difference in rates of GTP hydrolysis by TC10 versus Cdc42 is greatly exaggerated. However, high concentrations of p50GAP stimulate the rate of GTPase hydrolysis by TC10 by approximately 22-fold, compared with only a ninefold stimulation of the Cdc42 GTPase rate. Therefore, high concentrations of p50GAP will reduce the difference between the rates of inactivation of TC10 and Cdc42.

We have not tested the activity of Rho-family GEFs on TC10, but it has been reported that Dbl, a GEF specific for Cdc42 and RhoA, and Lbc, a GEF specific for RhoA, have no effect on the dissociation rate of GDP from TC10 [32,33]. Taken together, these results argue that the regulation of TC10 is distinct from that of other Rho-family members.

Figure 8



Interaction of Cdc42 effectors with TC10. (a) Yeast two-hybrid screen of a mouse embryonic (day 9) library. TC10(Q75L)ΔC, which lacks the carboxy-terminal prenylation motif, in bait vector pGBT9 was used to screen the library. Approximately 6.5×10^6 transformants were screened on selective medium, and colonies were tested in β-galactosidase assays. Plasmids were rescued from the yeast transformants, transformed into *E. coli*, and analyzed by PCR or DNA sequencing. To test the specificity of the yeast HF7c clones, conjugation assays were performed to the yeast strain W303 (MATα) transformed with TC10(T31N)ΔC, TC10(Q75L)ΔC, Cdc42(Q61L)ΔC, Rac1(G12V)ΔC, RhoA(G14V)ΔC, Rab3A(Q81L)ΔC or vector (pGBT9). The number of clones obtained for each protein is given. A total of 45 PAK clones were identified. (b) Conjugation tests for the interaction of MLK3 (amino acids 348–847) and WASP (70–502) proteins with TC10(Q75L)ΔC and Cdc42(Q61L)ΔC. Interactions were compared with those obtained for MLK2 (406–585) and N-WASP (140–269). SNF4 was used as a negative control for binding to Cdc42(Q61L)ΔC. (c) Overlay assay for interaction of GST–ACK-1 (504–545) protein with TC10 and Cdc42. Myc–αPAK was expressed in COS-7 cells as in Figure 7a. For GST–ACK-1 (Cdc42-binding domain), 1 μg was loaded per lane. GST (5 μg) was the negative control. Proteins were transferred to nitrocellulose and probed with equal specific activities of [α-^{32}P]GTP–TC10 or [α-^{32}P]GTP–Cdc42.

Because of sequence similarities in the effector domains of TC10 and other Rho-family members, we predicted that

TC10 may bind to an overlapping set of effectors. Indeed, TC10 interacts in a GTP-dependent manner with nine known effectors of Cdc42 and Rac: IQGAP1, α-, β- and γPAK, MRCKα/β, MLK2, N-WASP and MSE55. It also activates αPAK and JNK. TC10 did not interact with MLK3 or WASP, despite close similarities in the CRIB domains between these proteins and their relatives, MLK2 and N-WASP. TC10 did not bind appreciably to ACK-1. These differences in effector interactions may underlie the differences in phenotype induced by gain-of-function mutants of TC10 and Cdc42 and are likely to reflect important functional distinctions between them. The differences in interaction also provide an opportunity to dissect the features within the CRIB domains or their flanking regions that confer binding specificity. Finally, we note that the two-hybrid screen revealed several clones encoding novel potential TC10 and Cdc42 targets and others that interact only with TC10. The existence of these latter proteins supports our hypothesis that TC10 provides unique functions within the cells in which it is expressed.

Conclusions

TC10 is a distinct member of the Rho-family of GTPases and is expressed at high levels in muscle. Ectopic expression of gain-of-function TC10 mutants in fibroblasts produces a phenotype similar to that of Cdc42, but with exaggerated peripheral extensions and loss of stress fibers. The regulation of TC10 is complex because it has a lower intrinsic GTPase activity and lower affinity for p50GAP than Cdc42, but it has a greater increase in GTPase activity over basal levels in the presence of high GAP concentrations. Finally, although TC10 can associate with many of the same effectors as Cdc42 and Rac, it does not interact with MLK3 or WASP and interacts only weakly with ACK-1.

Materials and methods

Construction and expression of gene products

TC10 cDNA (gift from P. D'Eustachio, New York University) was amplified by PCR using primers that introduced 5′ *Bam*HI and 3′ *Eco*RI sites. Point mutations in TC10 were introduced by megaprimer PCR [34]. PCR products were inserted into pGEX-2T (Pharmacia). pGEX-Cdc42, pKH3-Cdc42(Q61L), pKH3-Rac1(G12V) and pKH3-RhoA(G14V) were gifts from R. Cerione, Cornell University. *TC10* and *Cdc42* were subcloned into *Bam*HI and *Eco*RI sites of pKH3 [35] and pKMyc mammalian expression vectors for production of proteins tagged with triple-HA or Myc, respectively. pKMyc vector was constructed by cloning the *Sal*I–*Eco*RI fragment of pCMV6M-αPAK (gift from G. Bokoch, Scripps) into the pRK7 vector [35]; αPAK was then removed by a *Bam*HI and *Eco*RI digest.

GST fusion proteins were produced in *E. coli* DH5α and purified on glutathione–Sepharose beads (Pharmacia). Proteins were eluted with glutathione or cleaved by thrombin (gift from P. Tracey, University of Vermont), which was removed with *p*-aminobenzamide–Sepharose (Sigma). Protein concentrations were quantitated by Bradford assays using BSA as a standard.

Cell culture and transfection

NIH3T3 and COS-7 cells were maintained in Dulbecco's modified Eagles medium containing 4.5 g/l glucose, 10% calf serum, and 1% penicillin/streptomycin. Cells were transfected by the $CaPO_4$ method [36]. Amounts of DNA transfected are noted in figure legends.

Immunofluorescence

Cells were fixed with paraformaldehyde [37] then permeabilized with 0.2% Triton X-100 for 2 min. HA-tagged proteins were detected with monoclonal 12CA5 (1:400) or polyclonal anti-HA (1:500) antibodies; Myc-tagged proteins were detected with monoclonal 9E10 anti-Myc antibody (1:1000). Secondary antibodies included Cy3-conjugated anti-mouse (1:1000), Texas-red-conjugated anti-mouse (1:500), or FITC-conjugated anti-rabbit (1:500) (Jackson ImmunoResearch). FITC-conjugated (1:100; Sigma) or TRITC-conjugated phalloidin (1:200; Molecular Probes) was used to stain F-actin. Fluorescence was visualized with a Nikon inverted microscope and a 60× water immersion lens. Cell phenotypes were quantitated by counting more than 100 cells in each of two experiments. Unpaired Student *t* tests were used to determine *p* values.

Northern blot

TC10, *Cdc42* or *β-actin* open reading frames were labeled with [α-^{32}P]dCTP (3000 Ci/mmol) by random priming (GibcoBRL) and used to probe a human poly(A)$^+$ RNA multiple tissue northern blot (Clontech) according to the manufacturers' instructions.

GTP release and hydrolysis assays

Recombinant GTPases (3–6 μg) were loaded with 5–10 μCi [α-^{32}P]GTP (3000 Ci/mmol) or [γ-^{32}P]GTP (5000 Ci/mmol) in 25 mM MOPS pH 7.1, 1 mM EDTA containing BSA (1 mg/ml) for 20 min on ice. Nucleotides were trapped by addition of $MgCl_2$ to 20 mM [38]. Bound nucleotides were measured by scintillation counting after nitrocellulose filter binding [39].

To measure GTP release and hydrolysis activities, ^{32}P-nucleotide-loaded TC10 or Cdc42 were diluted in GTPase buffer (25 mM MOPS pH 7.1, 1 mM EDTA containing 1 mM GTP, 1 mM GDP, 5 mM $MgCl_2$) and incubated at 30°C. At intervals, reactions were terminated by filter binding and quantitated by scintillation counting. The k_{off} values were calculated assuming single-exponential kinetics.

A GST fusion protein of the GAP domain of p50GAP (residues 205–439) (gift from Y. Zheng, University of Tennessee) was purified from *E. coli*. Reactions containing various concentrations of GST–p50GAP in GTPase buffer were initiated by addition of [γ-^{32}P]GTP-loaded TC10 or Cdc42, incubated at 30°C for 3 min, and terminated by filter binding. Intrinsic k_{cat} values were calculated and subtracted from k_{cat} values in the presence of GAP, assuming single-exponential kinetics. The apparent affinities of TC10 and Cdc42 for GST–p50GAP were estimated as the GAP concentrations yielding half-maximal acceleration of hydrolysis.

Overlay assays

Vector-transfected or pCMV6M-αPAK-transfected COS-7 cell lysates, 3 μg of Glu–Glu-tagged IQGAP1 (864–1657) (gift from R. Cerione) [40], 1 μg of GST–ACK-1 Cdc42-binding domain (504–545) (gift from E. Manser, National University of Singapore), or 5 μg of GST were fractionated by SDS–PAGE and transferred to nitrocellulose. An overlay assay was performed [38] with recombinant TC10, TC10(G26V) or Cdc42 loaded to equal specific activities with [α-^{32}P]GTP or [α-^{32}P]GDP. Protein interactions were visualized by autoradiography.

Protein kinase assays

For JNK assays, cells were co-transfected with pKH3-JNK and pKMyc-TC10(Q75L) or pKMyc-Cdc42(Q61L). For PAK assays, cells were transfected with pCMV6M-αPAK. At 24 h after transfection, cells were transferred to serum-free medium and starved overnight. Kinases were immunoprecipitated from cell lysates with 12CA5 or polyclonal anti-PAK (Santa Cruz) antibodies (1 h, 4°C) and protein A–Sepharose. Beads were washed three times with 2 mM sodium vanadate, 0.1% igepal in PBS, once with 0.5 M LiCl, 0.1 M MOPS pH 7.5, and once

with kinase buffer (12.5 mM MOPS pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM sodium vanadate). JNK reactions were initiated by addition of 2 μg GST–Jun (1–79) and 2 μCi [γ-^{32}P]ATP (6000 Ci/mmol). αPAK was stimulated with 5 μg TC10 or Cdc42 (loaded with 2 mM GTP) for 5 min on ice. αPAK reactions were initiated by addition of 5 μg MBP and 5 μCi [γ-^{32}P]ATP. After 20 min at 30°C, reactions were stopped by the addition of SDS–PAGE sample buffer. Phosphorylated substrates were visualized after SDS–PAGE by autoradiography. Protein expression was analyzed by immunoblotting with 12CA5 (1:7000) or 9E10 (1:2000) antibodies, horseradish peroxidase-conjugated anti-mouse antibody and chemiluminescence (Kirkegaard & Perry Laboratories).

Yeast two-hybrid screening

TC10(Q75L)ΔC, which lacks the carboxy-terminal prenylation motif (CCLIT), was subcloned into bait vector pGBT9 (Clontech) and used to screen a mouse embryo (day 9) library in pVP16 vector (gift from S.Hollenberg, Fred Hutchinson Cancer Research Center) [29]. Plasmids were co-transformed into *Saccharomyces cerevisiae* HF7c. Approximately 6.5×10^6 transformants were screened on synthetic dropout medium (Trp^- Leu^- His^-), containing 10 mM 3-amino triazole (3-AT) to reduce non-specific background. Colonies were tested for *lacZ* activity using X-gal (5-bromo-4-chloro-3-indolylβ-D-galactopyranoside; Sigma). Positives were tested for non-specific activation against empty pGBT9 vector and unrelated baits by conjugation assays. TC10(Q75L)ΔC and unrelated baits in pGBT9, or pGBT9 empty vector, were transformed into W303-B1(Y142; MATα) and grown overnight in the presence of each positive clone in HF7c (MATa). To test for conjugation, colonies were replica plated onto Leu^- Trp^- medium. To detect interaction, colonies were replica plated onto Leu^- Trp^- His^- +3-AT medium. Plasmids were then rescued from yeast transformants, transformed into *E. coli* DH5α and analyzed by PCR or DNA sequencing. pVP16 plasmids were retransformed into HF7c. Conjugation assays were performed to test the specificity of the interaction, using W303-B1(Y142) transformed with carboxy-terminal deletions of TC10(Q75L), TC10(T31N), Cdc42(Q61L), Rac1(G12V) or RhoA(G14V) in pGBT9. To test the interaction with MLK3 and WASP proteins, HF7c clones were co-transformed with one of the following plasmids: pACT-MLK3 (348–847), pACT-WASP (70–502) (gift from P. Aspenström, Ludwig Institute, Sweden), pACT-SNF4, pGBT9-MLK2 (406–585) or pGBT9-N-WASP (140–269) and either TC10(Q75L)ΔC or Cdc42(Q61L)ΔC. Colonies were randomly selected and grown on Leu^- Trp^- His^- +3-AT medium.

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References

1. Nobes CD, Lauritzen I, Mattei M-G, Paris S, Hall A, Chardin P: A new member of the Rho-family, Rnd1, promotes disassembly of actin filament structures and loss of cell adhesion. *J Cell Biol* 1998, 141:187-197.
2. Symons M: Rho-family GTPases: the cytoskeleton and beyond. *Trends Biochem Sci* 1996, 21:178-181.
3. Tapon N, Hall A: Rho, Rac, and Cdc42 GTPases regulate the organization of the actin cytoskeleton. *Curr Opin Cell Biol* 1997, 9:86-92.
4. Van Aelst L, D'Souza-Schorey C: Rho GTPases and signaling networks. *Genes Dev* 1997, 11: 2295-2322.
5. Keely PJ, Westwick JK, Whitehead IP, Der CJ, Parise LV: Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* 1997, 390:632-636.
6. Lacal JC: Regulation of proliferation and apoptosis by Ras and Rho GTPases through specific phospholipid-dependent signaling. *FEBS Lett* 1997, 410:73-77.
7. Drivas GT, Shih A, Coutavas E, Rush MG, D'Eustachio P: Characterization of four novel ***ras***-like genes expressed in a human teratocarcinoma cell line. *Mol Cell Biol* 1990, 10:1793-1798.
8. Ridley AJ, Hall A: The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 1992, 70:389-399.
9. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A: The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* 1992, 70:401-410.
10. Nobes CD, Hall A: Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 1995, 81:53-62.
11. Kozma R, Ahmed S, Best A, Lim L: The ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol Cell Biol* 1995, 15:1942-1952.
12. Prendergast GC, Khosravi-Far R, Solski PA, Kurzawa H, Lebowitz PF, Der CJ: Critcal role of Rho in cell transformation by oncogenic Ras. *Oncogene* 1995, 10:2289-2296.
13. Olson MF, Ashworth A, Hall A: An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science* 1995, 269:1270-1272.
14. Khosravi-Far R, Solski PA, Clark GJ, Kinch MS, Der CJ: Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for ras transformation. *Mol Cell Biol* 1995, 15:6443-6453.
15. Roux P, Gauthier-Rouviere C, Doucet-Brutin S, Fort P: The small GTPases Cdc42Hs, Rac1, and RhoG delineate Raf-independent pathways that cooperate to transform NIH3T3 cells. *Curr Biol* 1997, 7:629-637.
16. Qiu RG, Chen J, McCormick F, Symons M: A role for Rho in Ras transformation. *Proc Natl Acad Sci USA* 1995, 92:11781-11785.
17. Qiu R-G, Chen J, Kirn D, McCormick F, Symons M: An essential role for Rac in Ras transformation. *Nature* 1995, 374:457-459.
18. Minden A, Lin A, Claret FX, Abo A, Karin M: Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* 1995, 81:1147-1157.
19. Coso OA, Chiariello M, Yu J-C, Teramoto H, Crespo P, Xu N, *et al*.: The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* 1995, 81:1137-1146.
20. Bagrodia S, Derijard B, Davis RJ, Cerione RA: Cdc42 and PAK-mediated signaling leads to jun kinase and p38 mitogen-activated protein kinase activation. *J Biol Chem* 1995, 270:27995-27998.
21. Lamarche N, Tapon N, Stowers L, Burbelo PD, Aspenstrom P, Bridges T, *et al*.: Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of $p65^{PAK}$ and the JNK/SAPK MAP kinase cascade. *Cell* 1996, 87:519-529.
22. Burbelo PD, Drechsel D, Hall A: A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J Biol Chem* 1995, 270:29071-29074.
23. Yang W, Cerione RA: Cloning and characterization of a novel Cdc42-associated tyrosine kinase, ACK-2, from bovine brain. *J Biol Chem* 1997, 272:24819-24824.
24. Miki H, Sasaki T, Takai Y, Takenawa T: Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP. *Nature* 1998, 391:93-96.
25. Leung T, Chen X-Q, Tan I, Manser E, Lim L: Myotonic dystrophy kinase-related Cdc42-binding kinase acts as a Cdc42 effector in promoting cytoskeletal reorganization. *Mol Cell Biol* 1998, 18:130-140.
26. Manser E, Leung T, Salihuddin H, Zhao Z, Lim L: A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* 1994, 367:40-46.
27. Martin GA, Bollag G, McCormick F, Abo A: A novel serine kinase activated by rac1/CDC42Hs-dependent autophosphorylation is related to PAK65 and STE20. *EMBO J* 1995, 14:1970-1978.
28. Knaus UG, Morris S, Dong HJ, Chernoff J, Bokoch GM: Regulation of human leukocyte p21-activated kinases through G protein-coupled receptors. *Science* 1995, 269:221-223.
29. Vojtek AB, Hollenberg SM, Cooper JA: Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* 1993, 74:205-214.
30. Takano H, Komuro I, Oka T, Shiojima I, Hiroi Y, Mizuno T, *et al*.: The Rho-family G proteins play a critical role in muscle differentiation. *Mol Cell Biol* 1998, 18:1580-1589.
31. Erickson JW, Zhang C, Kahn RA, Evans T, Cerione RA: Mammalian Cdc42 is a brefeldin A-sensitive component of the Golgi apparatus. *J Biol Chem* 1996, 271:26850-26854.
32. Hart MJ, Eva A, Zangrilli D, Aaronson SA, Evans T, Cerione RA, *et al*.: Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. *J Biol Chem* 1994, 269:62-65.

33. Zheng Y, Olson MF, Hall A, Cerione RA, Toksoz D: Direct involvement of the small GTP-binding protein Rho in ***lbc*** oncogene function. *J Biol Chem* 1995, 270:9031-9034.
34. Barik S: Site-directed mutagenesis by double polymerase chain reaction: megaprimer method. In *PCR Protocols: Current Methods and Applications.* Edited by White BA. Totowa NJ: Humana Press; 1993:277-286.
35. Brondyk WH, McKiernan CJ, Fortner KA, Stabila P, Holz RQ, Macara IG: Interaction cloning of Rabin3, a novel protein that associates with the Ras-like GTPase Rab3A. *Mol Cell Biol* 1995, 15:1137-1143.
36. Sambrook J, Fritsch EF, Maniatis T: Expression of cloned genes in cultured mammalian cells. In *Molecular Cloning: A Laboratory Manual.* Edited by Nolan, C. Plainview, NY: CSH Press; 1989:16.32-16.36.
37. Carey KL, Richards SA, Lounsbury KM, Macara IG: Evidence using a green fluorescent protein-glucocorticoid receptor chimera that the Ran/TC4 GTPase mediates an essential function independent of nuclear protein import. *J Cell Biol* 1996, 133:985-996.
38. Lounsbury KM, Beddow AL, Macara IG: A family of proteins that stabilize the Ran/TC4 GTPase in its GTP-bound conformation. *J Biol Chem* 1994, 269:11285-11290.
39. Burstein ES, Macara IG: Characterization of a guanine nucleotide-releasing factor and a GTPase-activating protein that are specific for the ras-related protein p25rab3A. *Proc Natl Acad Sci USA* 1992, 89:1154-1158.
40. Hart MJ, Callow MG, Souza B, Polakis P: IQGAP1, a calmodulin-binding protein with a rasGAP-related domain, is a potential effector for cdc42Hs. *EMBO J* 1996, 15:2997-3005.

Supplementary material

Distinct cellular effects and interactions of the Rho-family GTPase TC10

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Movies

Movie 1 is a time-lapse video of peripheral extensions in cells expressing HA-tagged TC10(Q75L). NIH3T3 cells were transfected with 5 μg each of pRK7-GFP and pKH3-TC10(Q75L) on Eppendorf CELLocate gridded coverslips in 35 mm plates. Images of cells expressing GFP were captured and processed as in Figure 2.

Movie 2 is a time-lapse video of control cells expressing GFP. NIH3T3 cells were transfected with 5 μg pRK7-GFP and processed as above.